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Unraveling Viral Interleukin-6 Binding to gp130 and Activation of STAT-Signaling Pathways Independently of the Interleukin-6 Receptor▽‡

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Human herpesvirus 8 encodes a viral version of interleukin-6 (vIL-6) which shows 25% sequence homology with human IL-6. In contrast to human IL-6, which first binds to the IL-6 receptor (IL-6R) and only subsequently associates with the signal transducing receptor subunit gp130, vIL-6 has been shown to directly bind to gp130 without the need of IL-6R. As a functional consequence, vIL-6 can activate far more target cells in the body since all cells express gp130, but only cells such as hepatocytes and some leukocytes express IL-6R. We sought to understand which amino acid sequences within the vIL-6 protein were responsible for its ability to bind and activate gp130 independent of IL-6R. As a first approach, we constructed chimeric IL-6 proteins in which all known gp130 interacting sites (sites II and III) were sequentially transferred from vIL-6 into the human IL-6 protein. To our surprise, human IL-6 carrying all gp130 interacting sites from vIL-6 did not show IL-6R-independent gp130 activation. Even more surprisingly, the loop between helix B and C of vIL-6, clearly shown in the crystal structure not to be in contact with gp130, is indispensable for direct binding to and activation of gp130. This points to an IL-6R induced change of site III conformation in human IL-6, which is already preformed in vIL-6. These data indicate a novel activation mechanism of human IL-6 by the IL-6R that will be important for the construction of novel hyperactive cytokine variants.

Members of the hematopoietic interleukin-6 (IL-6) cytokine family bind to either a gp130 homodimer or heterodimers of gp130 and the signal transducing receptors (β-receptors) LIFR, OSMR, or WSX1. The β-receptors are activated upon binding of the cytokine. Some cytokines (IL-6, IL-11, IL-27, and ciliary neurotrophic factor [CNTF]) must first form a complex with their specific ligand binding α-receptors (IL-6R, IL-11R, EBI3, and CNTFR) before binding to β-receptors (41). Dimerization of gp130 with itself or with the appropriate β-receptor leads to activation of members of the JAK family of kinases and the STAT1 and STAT3 transcriptional activators (9, 38). Three interaction sites within the human IL-6 (hIL-6) protein have been identified, which are involved in the interaction of IL-6 with the IL-6R and two molecules of the signal transducing protein gp130. These sites have been named site I, site II, and site III, respectively (13).

Human herpesvirus 8 (HHV-8), also called Kaposi's sarcoma-associated herpesvirus, interferes with the body's immune response and causes Kaposi's sarcoma, a cancer of blood vessel cells that often occurs in subepidermal tissues or the mucous membrane. HHV-8 was first identified in Kaposi's sarcoma tissues of AIDS-infected persons (6). HHV-8 is also linked to the development of primary effusion lymphoma (5, 29) and multicentric Castleman's disease (39) and has been associated with multiple myeloma (36). HHV-8 infects predominantly endothelial and other mesenchymal cells, as well as B lymphocytes. Interestingly, the genome of HHV-8 encodes a homolog of hIL-6 called viral IL-6 (vIL-6) (30). The human and viral IL-6 proteins share only 25% sequence homology (30). In contrast to hIL-6, which binds to IL-6R before associating with gp130, vIL-6 binds and activates gp130 without the need of the IL-6R (15). The crystal structure of vIL-6 bound to the extracellular domain of gp130 has been solved and shows that two vIL-6 molecules interact with two molecules of gp130 and identifies the amino acid residues of site II and site III as being involved in the interaction between cytokine and receptor (8). Since there is no IL-6R present in the vIL-6/gp130 complex, site I is unoccupied in this crystal structure (8).

A soluble form of the IL-6R (sIL-6R) was found in various body fluids (16, 32). The sIL-6R, together with IL-6, stimulates cells that only express gp130 (14, 23), a process which is named trans-signaling (38). Furthermore, it has been shown that sIL-6R strongly sensitizes IL-6 responsive target cells (34). Trans-signaling cell types that are exclusively responsive to IL-6/IL-6R but not to IL-6 alone include early hematopoietic progenitor cells (35), endothelial cells (37), osteoclasts (42), smooth muscle cells (20), and neuronal cells (24, 25).

vIL-6 mimics a number of IL-6 activities, including stimulation of IL-6-dependent B-cell line growth (4) and activation of the JAK/STAT signal transduction pathway in HepG2 cells (27), and has been shown to stimulate cells by intracellular signaling (22, 26). Viral IL-6 protects virally infected cells from

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undergoing growth arrest and apoptosis or cell death, a strategy applied by the immune system to limit viral infection. vIL-6 modulates signaling of the antiviral factor alpha interferon, which prevents virus-infected cells from growing (7). Therefore, vIL-6 is a promising target for novel therapies directed against HHV-8-associated tumors and malignancies. Therefore, understanding the structure/function of vIL-6 will be useful for the development of novel neutralization strategies. Even though vIL-6 can still bind to the IL-6R, the involvement of the IL-6R in cellular responses to vIL-6 is not completely clear. Although vIL-6 responses have been shown to be blocked by neutralizing IL-6R monoclonal antibodies (4), other groups have questioned the requirement of IL-6R for vIL-6-induced activation of cells (27, 47). The crystal structure, however, clearly shows IL-6R-independent binding of vIL-6 to gp130 (8).

Recombinant soluble gp130Fc (sgp130Fc), consisting of the extracellular domain of human gp130 fused to the Fc region of human immunoglobulin G1 (IgG1) (18), was shown to specifically inhibit cellular responses via the IL-6/sIL-6R complex, while cellular responses triggered by IL-6 alone remained unaffected. The sgp130Fc protein can also be used to block vIL-6 induced signals (28).

In the present study we sought to identify the sites of vIL-6, which enable this protein to directly bind to gp130. We constructed chimeric proteins of hIL-6 and vIL-6, in which amino acid residues of vIL-6 identified to be in contact with gp130 in the crystal structure (8) were inserted into hIL-6. Surprisingly, a chimera, which carried all gp130 interacting sites from vIL-6 failed to bind to gp130 in the absence of the IL-6R. In contrast, transferring only amino acids from site III of vIL-6, together with the loop between helix B and C resulted in a chimeric protein with the same properties as vIL-6. Our results indicate an unexpected and previously unreported involvement of this BC loop in gp130 binding and activation.

MATERIALS AND METHODS

Cells and proteins. COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD, and Manassas, VA). Ba/F3 cells (murine pre-B cells) stably transfected with human gp130 (Ba/F3-gp130 cells) or human gp130 and the IL-6R (Ba/F3-gp130/IL-6R cells) have been previously described (12, 45). All cells were grown in Dulbecco's modified Eagle medium (DMEM) high-glucose culture medium (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal calf serum, penicillin (60 mg/liter), and streptomycin (100 mg/liter) at 37°C with 5% CO_2 in a humidified atmosphere. For the cultivation of Ba/F3-gp130 cells, the medium was additionally supplemented with 10 ng of Hyper-IL-6/ml, a fusion protein of IL-6 and sIL-6R connected by a flexible polypeptide linker (12). sgp130Fc is a fusion protein of the extracellular portion of gp130 and the Fc portion of IgG1 (18). The recombinant proteins IL-6, Hyper-IL-6, and sgp130Fc were expressed and purified as previously described (12, 18, 43). sgp130 was from R&D (Minneapolis, MN). The peptide resembling the BC loop (NH_2-EFGKSVINV-COOH) was from Biosynthan (Berlin, Germany). Anti-gp130 monoclonal antibody (MAb) BP-4 was from Diaclone (Hölzel Diagnostika GmbH, Cologne, Germany). The neutralizing anti-IL-6 MAb B-E8 was from Diaclone Research (Stanford, CT). Mouse anti-STAT3 MAb and rabbit anti-phospho-STAT3 MAb (Y705) were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-c-myc MAb 9E10 was from Abcam (Cambridge, United Kingdom). Peroxidase-conjugated ImmunoPure goat anti-rabbit IgG and peroxidase-conjugated ImmunoPure goat anti-mouse IgG were from Thermo Fisher. All restriction enzymes were from MBI Fermentas (St. Leon-Rot, Germany). [^{3}H]thymidine was purchased from Amersham Biosciences (Freiburg, Germany).

Molecular modeling. The boundaries of the IL-6 and vIL-6 regions exchanged were derived from the X-ray structures of vIL-6–gp130 and IL-6–IL-6R–gp130 complexes as taken from the Brookhaven data bank (accession code 1i1r and 1p9m, respectively). The interchanged regions are color coded on the complex models vIL-6–gp130 X-ray structures (see Fig. 1A). Structure comparisons and all computer graphic work were performed with the WHAT-IF program package running on an SGI-Indigo2 (46). For graphical representation, the program RIBBONS was used (31).

Cloning. The expression plasmid pet22b-IL-6-histag was generated by cloning the IL-6 PCR product (primers 5′IL-6 [5′-GTCACATATGCCAGTACCCCCA GGAG-3′] and 3′IL-6 [5′-GACTGCGGCCGCGGATCCCATTTGCCGAAGA GCCCTC-3′]) into pet22b using NdeI and NotI. pet22b was also used for the cloning of IV1, IV2, IV3, and IV4 using NdeI and NotI. pet22b-IL-6-histag was used as a template to generate pet22b-IV1-3. cDNAs coding for IV1, IV2, and IV3 were generated by splicing by overlapping extension PCR using the following primer combinations: T7 promoter and 5′siteIIb/3′siteIIb and T7 terminator to generate IV1; T7 promoter and 5′siteIIa/3′siteIIa and T7 terminator to generate IV2; T7 promoter and 5′siteIIa/3′siteIIa and 5′siteIIb/3′siteIIb and T7 terminator to generate IV3 (5′siteIIa [5′-CCAATTGAGTCTCTGAATGAGAAGTTC TGAAGAGGTGAGTG-3′], 3′siteIIa [5′-CTTCTCATTCAGAGACTCAATT GGATGCTATGGGGCATCTCAGCCCTGAG], 5′siteIIb [5′-GTCCCATCCT AAGGTCTTCGTCAGAAGCTCCATGACGTCGGCTTGTTCCTCACTACT C-3′]; 3′siteIIb [5′-GACGAAGACCTTAGGATGGGACCTGCAGAAAAAGG CAAAG-3′]). The cDNA coding for IV4 was generated by splicing by overlapping extension PCR using pet22b-IV3 as a template and the following primer combinations: T7 promoter and 5′siteIIIa (PCR-product6), 3′siteIIIa and 5′siteIIIb (PCR-product7), 3′siteIIIb and T7 terminator (PCR-product8) (5′siteIIIa [5′-CTAGAAT ACCCTTGCAGATGCCGGTACGGTAACAGAGGTCGCGGAAGCATTCAT CGATGCCCCATAGCATCCAATTGAGTCTCTG-3′]; 3′siteIIIa [5′-CATCTGC AAGGGTATTCTAGAGCCCGCTGCTATTTTTCATCTGAAACTTCCAAAG ATGGCTGAAAAAG-3′]; 5′siteIIIb [5′-AAAACGAAGCAAAGTGTCTCACC CAATACTTAAGTCCCTGCAGCTTCGTCAGCAG-3′]; 3′siteIIIb [5′-GAGAC ACTTTGCTTCGTTTTATGTTCTGAGTGCATTTAAGGAGTTCC-3′]). The resulting plasmids pet22b-IV1, pet22b-IV2, pet22b-IV3, and pet22b-IV4 were verified by sequence analysis and transformed into *E. coli* BL21/pLysS.

The plasmid pCR-Script IL-6-IV9 containing the codon-optimized cDNA coding for the hIL-6 chimera IV9 was synthesized as described by GeneArt (Regensburg, Germany). The IV9-coding regions of sites IIIa, IIIb, and IIIc were flanked by unique restriction endonuclease recognition sites to enable modular exchange of IL-6/vIL-6 sequences with self-hybridized oligonucleotides coding for IL-6 sites IIIa, IIIb, and IIIc. vIL-6-encoded site IIIa of IV9 was reverted into the original IL-6 sequence using the oligonucleotides 5′siteIIIa (5′-GCTCTGAGAAAGGAGACA TGTAACAAGAGTAACATGTGTGAAAGCAGCAAAGAGGCACTGGCAG AAAACAACCTGAACCT GCCAAA-3′) and 3′siteIIIa (5′-ATTTTTGGCAGGT TCAGGTTGTTTTCTGCCAGTGCCTCTTTGCTGCTTTCACACATGTTACT CTTGTTACATGTCTCCTTTCTCAGAGC-3′) and the restriction endonucleases AfeI and BspMI. vIL-6-encoded site IIIb of IV9 was reverted to the original IL-6 sequence by using the oligonucleotides 5′siteIIIb (5′-GGCACAGAACCAGTG GCTGCAGGACATGACAACTCATCTCATTCTGCGCAGCTTCAAAG-3′) and 3′siteIIIb (5′-AATTCTTTGAAGCTGCGCAGAATGAGATGAGTTGTC ATGTCCTGCAGCCA CTGGTTCTGTGCCTGCA-3′) and the restriction endonucleases SalI and BstBI. vIL-6-encoded site IIIa of IL-6–IV9 was reverted to the original IL-6 sequence by using the oligonucleotides 5′siteIIIc (5′-CGAAG TGTACCTGGAATACCTGCAGAACAGATTTGAGAGTAGTGAGGAAC AAGCCAGAGCTGTGCAGATG-3′) and 3′siteIIIc (5′-TCGACATCTCTGC ACAGCTCTGGCTTGTTGTTCCTCACTACTCTCAAATCTGTTCTGCAG GTATTCCAGGTA-3′) and the restriction endonucleases PstI and EcoRI. The original plasmid pCR-Script-IV9 and the newly generated plasmids pCR-Script-IV5, pCR-Script-IV6, pCR-Script-IV7, and pCR-Script-IV8 were digested with NdeI and NotI. The cDNAs coding for IV5 to IV9 were cloned into the respective sites of pESL-IL-6-c-myc-histag (unpublished results) to obtain the plasmids pESL-IV5-c-myc-histag, pESL-IV6-c-myc-histag, pESL-IV7-c-myc-histag, pESL-IV8-c-myc-histag, and pESL-IV9-c-myc-histag. Thereby, the coding regions of IV5-IV9 were fused to the original signal peptide coding sequence of IL-6 and to a c-myc and His tag coding sequence at the 3′ end. The cDNAs were subsequently transferred into pcDNA3.1-DEST40 by using the Gateway system technology according to manufacturer's instructions (Invitrogen, Karlsruhe, Germany). The resulting plasmids pcDNA-DEST40-IV5, pcDNA-DEST40-IV6, pcDNA-DEST40-IV7, pcDNA-DEST40-IV8, and pcDNA-DEST40-IV9 were verified by sequence analysis and used for transient transfection of COS-7 cells. The alignments and amino acid sequences of the vIL-6, IL-6, IV4, and IV9 proteins are depicted in Fig. S1 to S3 in the supplemental material and were as follows: IV1 (the transfer of part of the C-helix from vIL-6 to IL-6: amino acids [aa] 134 to 153 of IL-6 exchanged to aa 116 to 135 of vIL-6), IV2 (the transfer of part of the A-helix from vIL-6 to IL-6: aa 52 to 62 of IL-6 exchanged to aa 34 to 44 of vIL-6), IV3 (the transfer of part of the A and C helices from vIL-6 to IL-6: aa 52 to 62 and 134 to 153 of IL-6 exchanged to

aa 34 to 44 and aa 116 to 135 of vIL-6, respectively), IV4 (in addition to IV3, transfer of the AB loop and part of the D-helix from vIL-6 to IL-6: aa 65 to 91 and aa 181 to 197 of IL-6 exchanged to aa 47 to 73 and aa 163 to 179 of vIL-6, respectively), IV5 (transfer of the BC loop from vIL-6 to IL-6: aa 130 to 142 of IL-6 exchanged to aa 112 to 124 of vIL-6), IV6 (transfer of the BC loop and part of the D-helix from vIL-6 to IL-6: aa 130 to 142 and aa 181 to 194 of IL-6 exchanged to aa 112 to 124 and aa 163 to 176 of vIL-6, respectively), IV7 (transfer of the AB loop and part of the D-helix from vIL-6 to IL-6: aa 67 to 89 and aa 181 to 194 of IL-6 exchanged to aa 49 to 71 and aa 163 to 176 of vIL-6, respectively), IV8 (transfer of the AB loop and the BC loop from vIL-6 to IL-6: aa 67 to 89 and 130 to aa 142 of IL-6 exchanged to aa 49 to 71 and aa 112 to 124) of vIL-6, respectively) and IV9 (transfer of the AB loop, the BC loop and part of the D-helix from vIL-6 to IL-6: aa 67 to 89, aa 130 to 142, and aa 181 to 194 of IL-6 exchanged to aa 49 to 71, aa 112 to 124, and aa 163 to 176 of vIL-6, respectively).

Preparation and quantification of recombinant proteins. *E. coli* BL21/pLysS cells were transformed with the expression vector pet22b-IV1-4. Transformed bacteria were grown to an A_{600} of approximately 0.5 to 0.7, and protein expression was induced by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After 2 h, isolation of inclusion bodies and renaturation was performed as described previously (43). Renaturation of proteins was achieved by dialysis against refolding buffer (1 M guanidinium chloride, 3 mM oxidized glutathione, and 0.6 mM reduced glutathione for 12 h and 20 mM Tris-HCl [pH 6.8] for 12 h). The purity of the recombinant proteins was ascertained by sodium dodecyl sulfate (SDS)–15% polyacrylamide gel electrophoresis and staining with Coomassie blue. Protein concentrations were determined by recording absorption spectra in the range from 240 to 320 nm, applying the method of Waxman et al. (48).

Transfection. COS-7 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfection efficiency, as visualized after 24 h by green fluorescent protein expression using Axiovert 200 Microscope (Zeiss), was ca. 80%. Cytokine activity assays were performed on centrifuged, sterile-filtered conditioned supernatants.

Immunoblotting and enhanced chemiluminescence detection. For Western blotting, proteins separated by SDS-polyacrylamide gel electrophoresis were transferred to polyvinylidene difluoride membranes (Hybond-P; Amersham Biosciences) by using a semidry electroblotting procedure. Membranes were blocked in a solution of TBS (10 mM Tris [pH 8], 150 mM NaCl) supplemented with 0.05% Tween 20 and 3% skimmed milk powder or 5% bovine serum albumin and then probed overnight with the indicated antibodies at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were detected by using a chemiluminescence kit (ECL Plus Western blotting detection system; Amersham Biosciences) according to the manufacturer's instructions.

Proliferation assays. Ba/F3–gp130–IL-6R cells were washed three times with sterile phosphate-buffered saline and resuspended in DMEM containing 10% fetal calf serum at 5×10^3 cells per well of a 96-well plate. The cells were cultured in a final volume of 100 μl with additional cytokines as indicated. Cells were subsequently pulse-labeled with 0.25 μCi of [^{3}H]thymidine for 4 h. The specific activity of [^{3}H]thymidine was 25 Ci/mmol. Cells were harvested on a glass fiber filter (Filtermat A; Wallac, Turku, Finland) and microwave baked for 2 min. Subsequently, the filter was soaked in 4.5 ml of liquid scintillation cocktail (Betaplate Scint; Wallac). [^{3}H]thymidine incorporated into cellular DNA was determined by scintillation counting using a MicroBeta TriLux counter from Perkin-Elmer (Wellesley, MA). Alternatively, a CellTiter-Blue cell viability assay (Promega, Mannheim, Germany) was used to determine the cell number according to the manufacturer's instructions and measured on a Lambda Fluoro 320 fluorimeter (excitation filter, 530/25 nm; emission filter, 590/35 nm; sensitivity 75; Software KC4).

Immunoprecipitation. Conditioned supernatant containing IV9 protein was incubated with 20 μg of sgp130Fc and 200 ng of sIL-6R in DMEM overnight at 4°C followed by the addition of 30 μl of protein A-Sepharose (50% slurry; CL-4B; Amersham Biosciences) for at least 4 h at 4°C. Immunoprecipitates were washed three times with washing buffer I (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.2% IGEPAL, 2 mM EDTA) and twice with washing buffer II (10 mM Tris-HCl [pH 7.6], 500 mM NaCl, 0.2% IGEPAL, 2 mM EDTA) prior to the addition of Laemmli sample buffer (2× Laemmli: 100 mM Tris [pH 6.8], 4% [wt/vol] SDS, 200 mM dithiothreitol, 20% [vol/vol] glycerol, 0.1% bromophenol blue) and boiling at 95°C for 5 min.

RESULTS

Transfer of the gp130 binding sites from vIL-6 to hIL-6. The crystal structure of vIL-6 bound to the extracellular portion of gp130 showed two distinct binding sites on the vIL-6 protein (Fig. 1A) (8). Site II consists of residues of helices A and C, whereas site III is composed of residues at the top of helix A, the beginning of the AB loop, the end of the CD loop, and the top of helix D (for details, see Materials and Methods). Even though Fig. 1A to C illustrates only vIL-6 in complex with gp130, the loop between helices B and C of vIL-6 and of IL-6 are not in contact to gp130 and is more than 15 Å away from gp130 (see Fig. S4A, B, and C in the supplemental material).

A series of four chimeric proteins was constructed by inserting the coding region for residues involved in binding of vIL-6 to gp130 into hIL-6 cDNA. As shown in Fig. 2A, the two epitopes forming site II were first transferred separately in chimeras IV1 and IV2. IV1 and IV2 were combined in chimera IV3 and in chimera IV4 with the two binding sites to gp130 of site III. The chimeric cDNAs were expressed in *E. coli*, purified, and refolded from inclusion bodies as described previously (11, 43). The purified proteins migrated as single bands upon SDS-gel electrophoresis (data not shown).

The biological activity of the chimeric proteins was tested on murine Ba/F3 pre-B cells. These cells proliferate in the presence of the IL-3. After stable transfection with a cDNA encoding gp130, these cells (Ba/F3-gp130) grow in the absence of IL-3 but in the presence of IL-6/sIL-6R, Hyper-IL-6 (a fusion protein of IL-6 and sIL-6R) (12), or viral IL-6 (28). Ba/F3-gp130 cells stably transfected with an IL-6R cDNA (Ba/F3-gp130/IL-6R cells) proliferate in the presence of IL-6 (18). Cell surface expression of gp130 and IL-6R in the stably transfected BAF/3 cells have been confirmed by fluorescence-activated cell sorting analysis (45, 2). As shown in Fig. 2B, hIL-6 requires IL-6R for stimulation of the cells, whereas Hyper-IL-6 does not. All four chimeric proteins failed to activate gp130 directly but were active in the presence of IL-6R, indicating that the exchanged residues, which in IV4 consist of both complete binding sites to gp130, are not sufficient to render the chimera independent of IL-6R. Although the chimera IV4 was 10-fold less active than IV1 to IV3, this result also shows that chimeras 1 to 4 are folded correctly, since they all are biologically active on Ba/F3-gp130/IL-6R cells.

Transfer of the extended site III gp130 binding site from vIL-6 to hIL-6. In a previous study we constructed chimeras of hIL-6 and CNTF that led to the identification of the CNTF region responsible for binding to the LIFR (19). Prior to the solving of the crystal structure of vIL-6 bound to gp130 (8) we speculated that the BC loop of human CNTF was part of the binding site III to LIFR. The reason for this speculation was that, in addition to residues at the top of helix A, the beginning of the AB loop, the end of the CD loop, and the top of helix D, residues forming the BC loop of CNTF had to be transferred into hIL-6 to obtain a chimeric protein able to bind to a receptor combination of IL-6R, gp130, and LIFR with activity similar to that of LIF (19). We speculated that as in CNTF for binding to LIFR, the BC loop of vIL-6 contributed to the binding site III of vIL-6 to gp130.

Therefore, as shown in Fig. 3A, we constructed a new series of chimeric proteins, in which we transferred the two sites constituting site III together with the residues forming the loop between helix B and C. Since the chimeric cDNAs could not be expressed in bacteria (data not shown), the cDNAs fused with the coding region for the signal peptide of hIL-6 at the 5′ end and the coding region for a c-myc tag, followed by a His_6 tag at

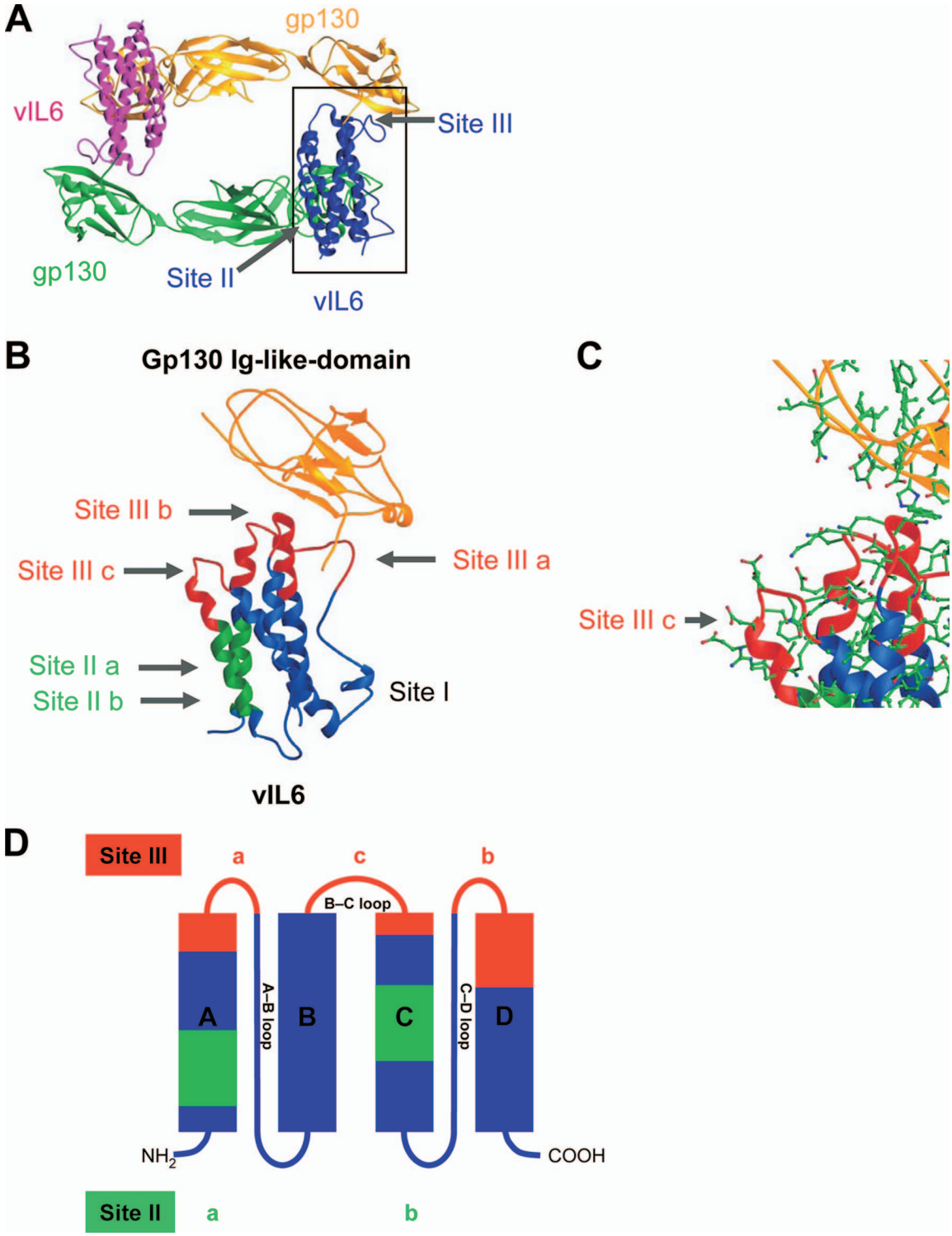

FIG. 1. Binding of vIL-6 to gp130. (A) Ribbon model of the recently solved vIL-6/gp130 X-ray structure. (B) Interaction of gp130 (only immunoglobulinlike domain D1) and vIL-6. Sites II and III are indicated. Detailed sequence information is shown in supplemental Fig. 1. (C) The BC loop of site III (site IIIc) is not in direct contact to gp130-D1. (D) Schematic drawing of the common four-helix bundle cytokine fold. The different parts of site II and site III are color coded as green (site II) or red (site III).

the 3′ end, were cloned into a mammalian expression plasmid. For increased expression in mammalian cells, the cDNAs were codon optimized without changing the amino acid sequence encoded by the chimeric constructs. The resultant expression plasmids were transiently expressed in COS-7 cells. The recombinant proteins were detected by Western blotting showing comparable expression levels of IV5 to IV9, IL-6, and vIL-6 (Fig. 3B).

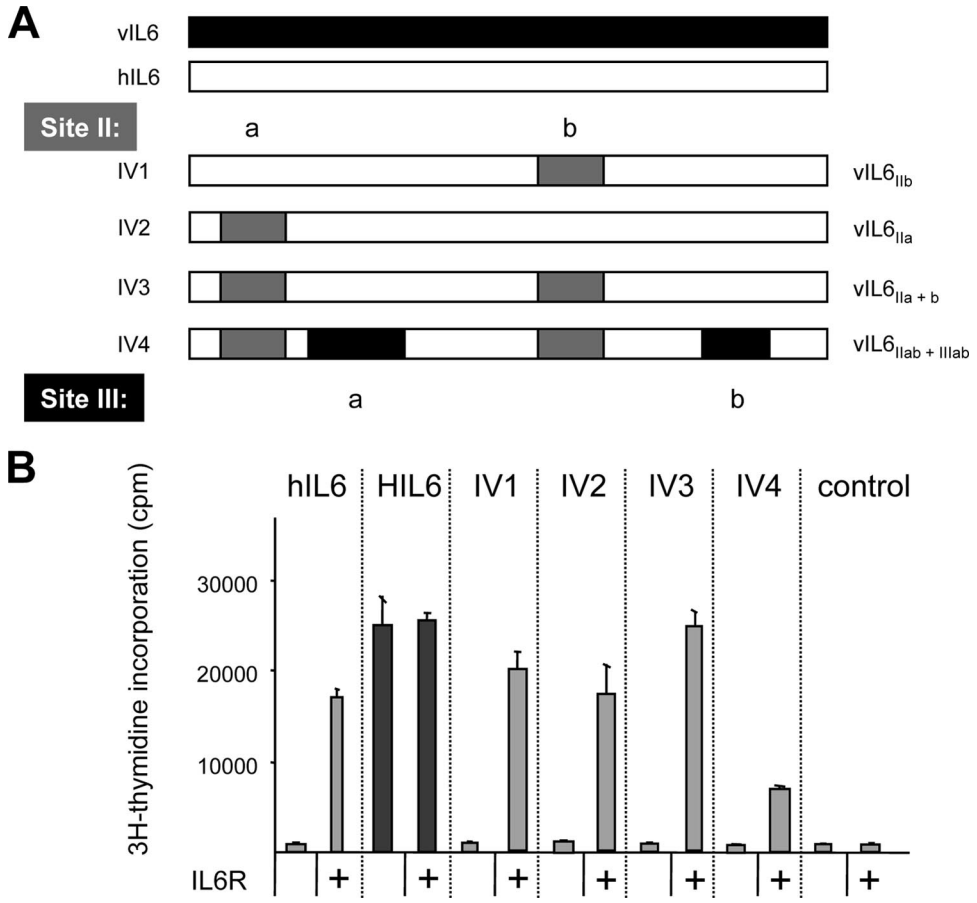

FIG. 2. Characterization of the vIL-6/hIL-6 chimeras IV1-4. (A) Schematic representation of vIL-6, hIL-6, and chimeras IV1 to IV4. Sequence stretches that are part of the exchanged areas of vIL-6 and hIL-6 are indicated in gray (site II) and black (site III). Detailed sequence information is shown in supplemental Fig. 2. (B) Equal numbers of stably transfected Ba/F3-gp130 or Ba/F3-gp130/IL-6R cells were cultured for 3 days in the presence of IL-6 (hIL6; 100 ng/ml), Hyper-IL-6 (HIL6; 100 ng/ml), IV1 (100 ng/ml), IV2 (100 ng/ml), IV3 (100 ng/ml), or IV4 (1,000 ng/ml). Proliferation was measured by pulse-labeling the cells after 72 h with [^{3}H]thymidine for 4 h. Cells were harvested, and the incorporated radioactivity was measured by scintillation counting. Bioassays were performed, with each value being determined in triplicate.

As shown in Fig. 3C, the chimeras IV5, IV6, IV7, and IV8 failed to stimulate Ba/F3-gp130 cells. However, when the sIL-6R was added to the chimeric proteins, all chimeras stimulated proliferation of the cells. Comparable results were obtained using Ba/F3-gp130/IL-6R cells (data not shown), clearly indicating that the chimeric proteins were properly folded and were biologically active. In contrast, the chimeric protein IV9 which contained the original gp130 contact site III, together with the BC loop of vIL-6, stimulated the proliferation of BaF/3-gp130 cells in both the absence and the presence of the sIL-6R protein, indicating that the BC loop of the vIL-6 protein is responsible for the IL-6R-independent gp130 interaction.

As an additional control, we incubated cells with the chimeras in the presence or absence of a hIL-6 neutralizing antibody and the sgp130Fc protein, which is known to bind to and inhibit the activity of vIL-6 and the IL-6/sIL-6R complex, but not the IL-6/membrane-bound IL-6R complex (18, 28). As shown in Fig. 4, the proliferation of Ba/F3-gp130 cells stimulated with vIL-6, which was independent of the sIL-6R, was blocked by the sgp130Fc protein but not by the neutralizing antibody against hIL-6. In contrast, hIL-6-stimulated Ba/F3-gp130 cells proliferate only in the presence of the sIL-6R. Both sgp130Fc and the anti-IL-6 antibody were able to inhibit this proliferation. Importantly, proliferation induced by chimera IV9 in the presence or absence of IL-6R could also be inhibited by both the anti-IL-6 antibody and the sgp130Fc protein, indicating that the epitope recognized by the antibody was still present in chimera IV9.

Since the protein IV9 could not be expressed in *E. coli* and attempts to purify the recombinant protein from cell culture supernatant failed, comparison of the biological activity (50% inhibitory concentration) of vIL-6 and IV9 was not possible. Nevertheless, from these qualitative assays we estimate that the biological activity of vIL-6 and IV9 was in the same range, since the COS-7 production levels of vIL-6 and IV9 were comparable, and equal amounts were used (data not shown).

vIL-6 is N glycosylated at N78 and N89. Dela-Cruz et al. showed that N glycosylation at site N89 of vIL-6 is required for enhanced binding of vIL-6 to gp130. hIL-6 is N glycosylated at N73 and O glycosylated at T166 and T170/T171, but neither N-linked nor O-linked glycosylation is necessary for IL-6R-dependent binding to gp130 (10). Importantly, none of the glycosylation sites of vIL-6 were transferred to IL-6 in the

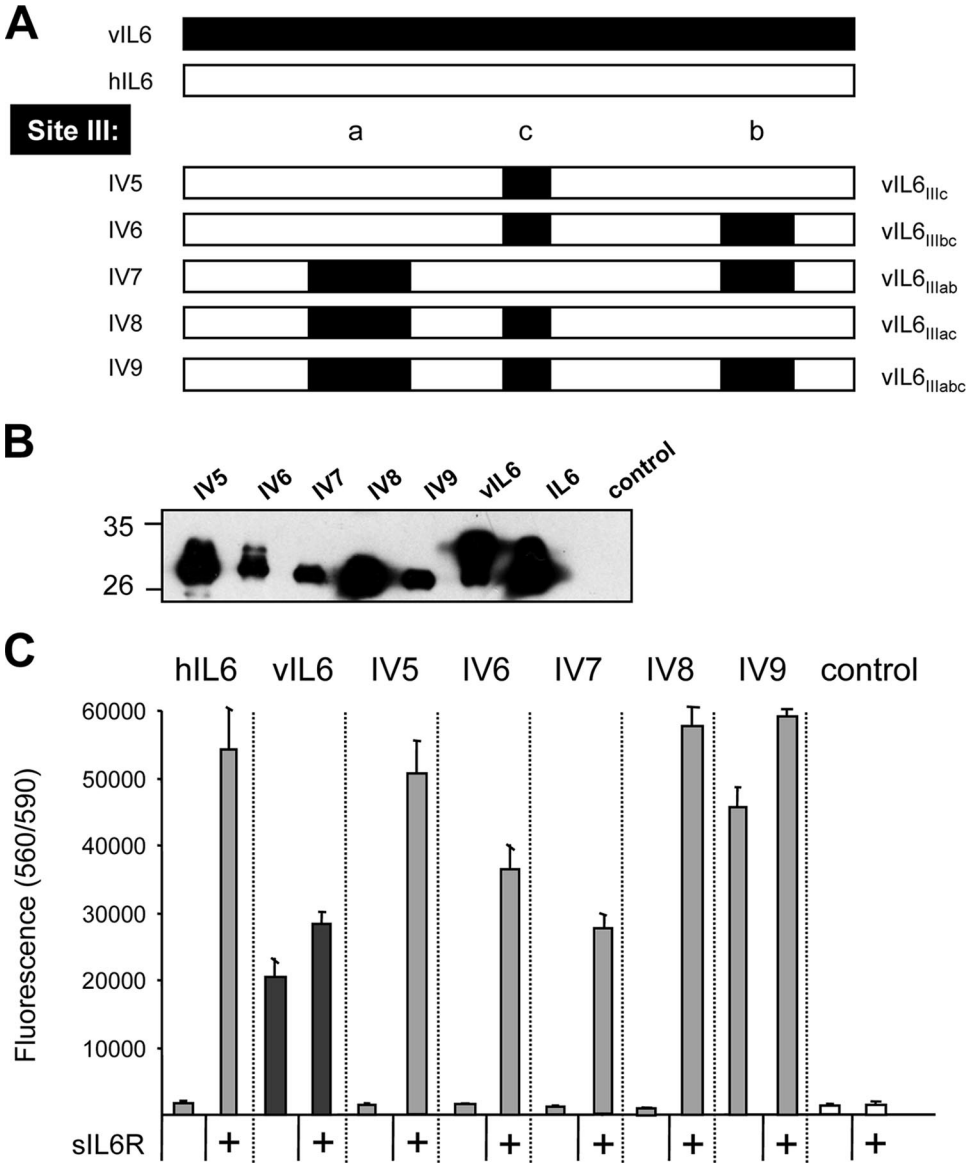

FIG. 3. Characterization of the vIL-6/hIL-6 chimeras IV5 to IV9. (A) Schematic representation of vIL-6, hIL-6, and chimeras IV5 to IV9. Sequence stretches that are part of the exchanged areas of vIL-6 and hIL-6 are indicated in black. Detailed sequence information is shown in supplemental Fig. 3. (B) Western blot analysis of supernatants of COS-7 cells transiently transfected with hIL-6, vIL-6, and IV5 to IV9 using anti-c-myc antibodies. (C) Equal numbers of stably transfected Ba/F3-gp130 cells were cultured for 3 days with conditioned supernatant containing hIL-6, vIL-6, IV5, IV6, IV7, IV8, or IV9 in the presence or absence of sIL-6R (1 μg/ml). Proliferation was measured by using a CellTiter-Blue cell viability assay. Bioassays were performed, with each value being determined in triplicate.

IL-6R-independent chimera IV9, indicating that IL-6R-independent binding of IV9 to gp130 is not based on N glycosylation of viral IL-6-N89 (see Fig. S3 in the supplemental material). Moreover, the crystal structure of gp130 in complex with vIL-6 was prepared with nonglycosylated proteins expressed in the presence of tunicamycin (8), providing another hint that glycosylation is not mandatory for gp130-engagement of vIL-6. Therefore, we conclude that binding of IV9 to gp130 is not influenced by glycosylation.

To analyze IV9-induced STAT3 phosphorylation, we used NIH 3T3 cells, which express gp130 but not IL-6R and therefore do not respond to IL-6 alone (Fig. 5A). IV9 induced STAT3 phosphorylation in the presence or absence of sIL-6R, although the presence of the sIL-6R led to increased STAT3 phosphorylation in comparison to IV9 alone, an observation also seen in Ba/F3-gp130 proliferation assays. These observations might indicate that the binding of IV9 to the IL-6R enhanced the biological activity of this chimera, which very likely was caused by the original IL-6R binding site from hIL-6 in the chimera IV9. As a control, the trans-signaling antagonist sgp130Fc, able to block IV9-induced STAT3 phosphorylation, was included.

To prove the direct physical interaction of IV9 and gp130 without the need of the sIL-6R, we performed coimmunoprecipitation experiments. IV7 was incubated with sgp130Fc in the presence or absence of the sIL-6R, and the sgp130Fc protein

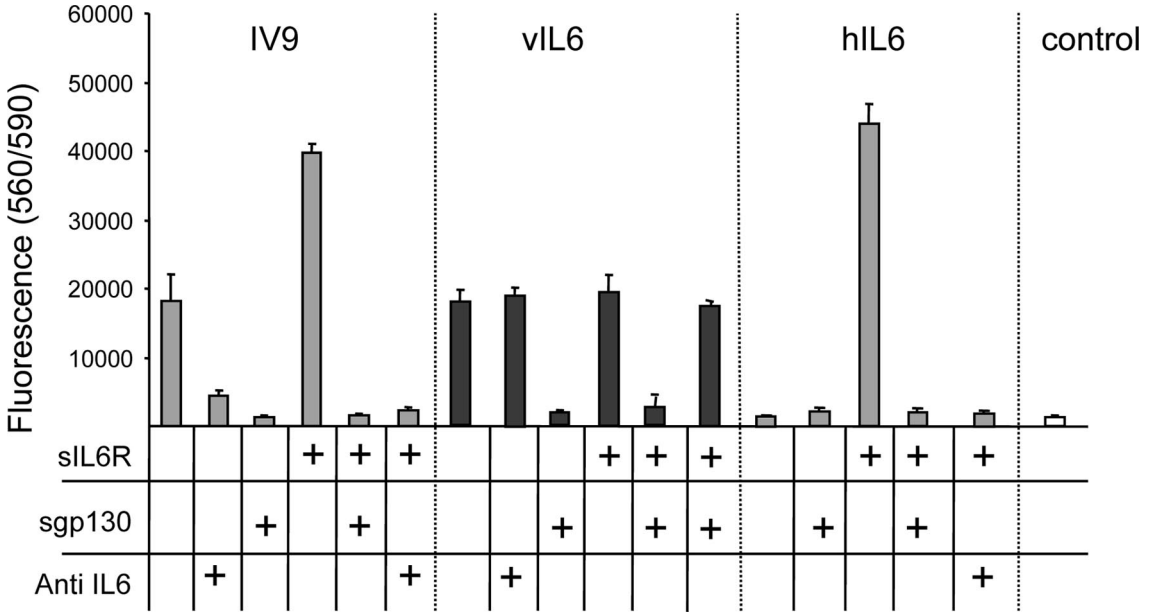

FIG. 4. IV9 was specifically inhibited by anti-IL-6 MAb and sgp130Fc. Equal numbers of stably transfected Ba/F3-gp130 cells were cultured for 3 days with conditioned supernatant containing hIL-6, vIL-6, or IV9 in the presence or absence of sIL-6R (1 μg/ml), sgp130Fc (10 μg/ml) or anti-IL-6 MAb (10 μg/ml). Proliferation was measured by using a CellTiter-Blue cell viability assay. Bioassays were performed, with each value being determined in triplicate.

was subsequently precipitated with protein A-Sepharose. The IV7 proteins were detected with anti-c-myc MAbs. As depicted in Fig. 5B (right panel), only IV9 was precipitated with sgp130Fc in the presence or absence of the sIL-6R, but not without sgp130Fc, demonstrating a direct interaction between IV9 and gp130, which is independent of the sIL-6R. IV7 could only be precipitated in the presence but not in the absence of the sIL-6R, indicating that this protein, which carried the AB loop and the D-helix from vIL-6 but not the BC loop from vIL-6 still needed the sIL-6R for the interaction with gp130. As an additional control, sgp130 was precipitated with c-myc-tagged IV9 by anti-c-myc tag antibodies (Fig. 5C).

We showed that the chimera IV5 that only contained the BC loop region of vIL-6 but not the AB loop and D-helix region of vIL-6 was not able to bind the gp130 independently of IL-6R. We showed in Fig. 1 that the BC loop is not directly interacting with gp130. To further substantiate this point, we used the peptide forming the BC loop of vIL-6 and performed the immunoprecipitation of IV9 and sgp130Fc in the presence of 100 μM BC peptide. In Fig. 5D, we show that the BC-forming peptide is not able to inhibit the complex formation between gp130 and IV9, again demonstrating that the BC loop is not directly interacting with gp130. This supports our hypothesis that the BC loop locks the AB loop and D-helix in a conformation that is able to bind to gp130 independently of IL-6R.

DISCUSSION

vIL-6 encoded by the HHV-8 belongs to the class of virokines, soluble secreted cytokine or chemokine homologues acquired from the human genome by molecular piracy (21). These proteins do not necessarily display high sequence homology to their human predecessors and often have very distinct functional and molecular capacities. hIL-6 and vIL-6 proteins exhibit only 25% sequence identity but share the same structural topology (7, 30). hIL-6 first binds to the IL-6R before associating with gp130, whereas vIL-6 binds and activates gp130 without the need of the IL-6R (15). The crystal structure of vIL-6 in complex with the three membrane distal domains of gp130 showed two vIL-6 molecules interacting with two molecules of gp130 via site II and site III (8). The crystal structure of IL-6 in complex with IL-6R and gp130 revealed that two IL-6 molecules interact with two molecules of gp130 via site II and site III and with two IL-6R molecules via site I (3). Since there was no IL-6R present in the vIL-6/gp130 complex, site I of vIL-6 remained unoccupied (8). Since vIL-6 is still able to bind to IL-6R via site I, but IL-6R is not mandatory for gp130 activation, one might speculate that a common ancestor displayed activities like the hIL-6 and not like vIL-6. Therefore, we assume that the IL-6R-independent binding of vIL-6 to gp130 is a unique event in vIL-6 evolution.

The precise role of vIL-6 in HHV-8 pathogenesis is not well understood. vIL-6 may act as an antiapoptotic factor for the survival of HHV-8-infected cells and/or promote the proliferation of HHV-8-infected cells or potential host cells. The IL-6R-independent binding of vIL-6 to gp130 forms the basis of the pathogenic significance of HHV-8, highlighted by the study by Chatterjee et al. (6), in which vIL-6 was shown to prevent HHV-8-infected cells from cellular death. Here, HHV-8-infected cells are targeted by the major antiviral immune mechanism induced by interferons, which mediates downregulation of the IL-6R but not of gp130. Thereby, antiapoptotic and mitogenic pathways in HHV-8-infected cells, which were induced by IL-6 via the membrane bound IL-6R, were abrogated and the cells undergo apoptosis. Expression of vIL-6 from the HHV-8-infected cell compensates for the loss of the IL-6R by

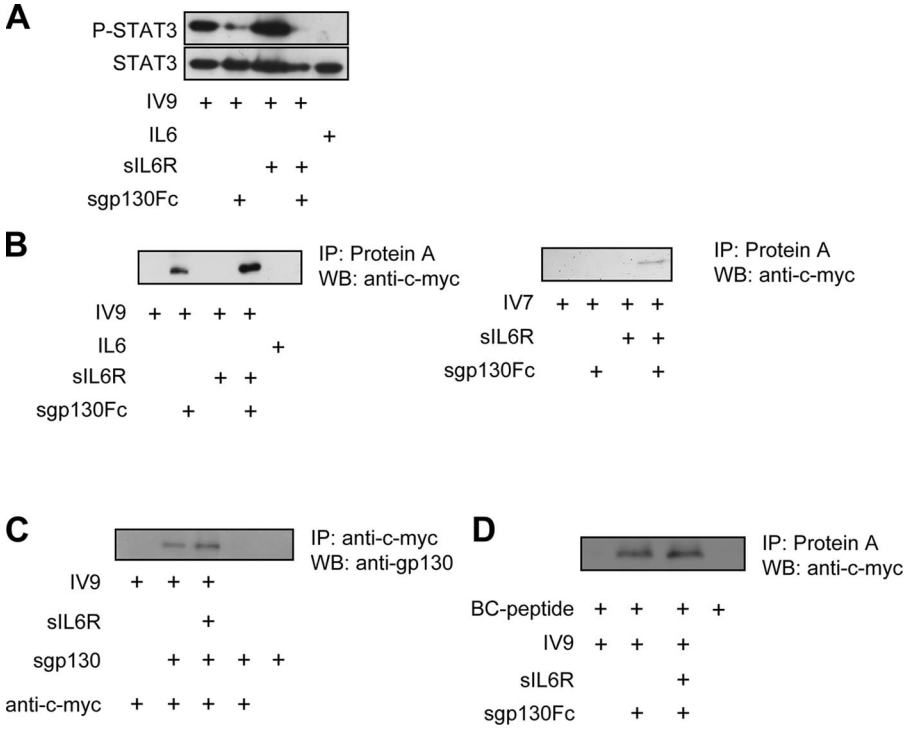

FIG. 5. IV9-induced STAT3 phosphorylation and coimmunoprecipitated of IV9 by sgp130Fc. (A) NIH 3T3 cells were stimulated with conditioned supernatant containing IV9 for 5 min in the presence or absence of sgp130Fc (10 μg/ml) or anti-IL-6 MAb (10 μg/ml). Unstimulated cells were used as controls. STAT3 phosphorylation was detected by Western blotting with anti-phospho-STAT3 specific antibodies. Western blotting against STAT3 served as a loading control. (B) Conditioned supernatant containing IV7 and IV9 was incubated with or without recombinant sgp130Fc protein in the presence or absence of sIL-6R and precipitated with protein A-Sepharose. Precipitated c-myc-tagged IV9, IV7, and IL-6 protein was detected by Western blotting with anti-c-myc specific antibodies. (C) Conditioned supernatant containing IV9 was incubated with or without recombinant sgp130 protein in the presence or absence of sIL-6R and precipitated with anti-c-myc tag antibodies. Precipitated sgp130 protein was detected by Western blotting with the anti-gp130 specific antibody BP-4. (D) Conditioned supernatant containing IV9 was incubated with or without recombinant sgp130Fc protein in the presence or absence of sIL-6R and the BC-forming peptide at a final concentration of 100 μM and precipitated with protein A-Sepharose. Precipitated c-myc-tagged IV9-protein was detected by Western blotting with anti-c-myc specific antibodies.

inducing auto- or paracrine gp130 signal transduction and antiapoptosis and mitogenesis even in the absence of IL-6R (7).

The molecular basis for the IL-6R-independent-binding of vIL-6 to gp130 is not understood, so we intended to generate an IL-6 molecule which was able to signal through gp130 without requiring the IL-6R. Based on the vIL-6/gp130 and IL-6/IL-6R/gp130 crystal structures, we constructed a variety of chimeric proteins, in which amino acid residues from vIL-6 were transferred to hIL-6 (8). These exchanges were composed of site II and site III from vIL-6, which were identified to be in direct contact with gp130. The chimeras IV1 to IV4 and the chimeras IV6 to IV8 containing site II and/or site III of vIL-6 were not able to induce IL-6R-independent gp130 activation. However, these chimeras were all biologically active in the presence of IL-6R, indicating the modular assembly of IL-6 and vIL-6, which allowed the step-by-step exchange of amino acid building blocks without affecting overall cytokine structure and function. The ability of all of these chimeras to induce a biological response, clearly demonstrate the promiscuity and little specificity of site II for the interaction with gp130.

A modular structure of a receptor recognition site was also demonstrated for IL-6 and CNTF, a cytokine that signals like LIF, but requires the CNTFR for binding to a gp130-LIFR heterodimer (19). Here, the exchange of site III from CNTF to IL-6 resulted in a cytokine, which bound to and activated a gp130/LIFR/IL-6R complex instead of a gp130/LIFR/CNTFR complex.

In the IL-6/vIL-6 chimera IV9 we transferred an extended site III from vIL-6 to IL-6, which consists of amino acid residues located in the site IIIa (C-terminal A helix, N-terminal AB loop), site IIIb (C-terminal CD loop with adjoining N-terminal D helix), and the site IIIc (BC loop with adjacent parts of the B and C helixes). Interestingly, IV9 induced STAT3 phosphorylation and cell proliferation of a cell line normally dependent on IL-6/sIL-6R. Furthermore, IV9 protein could be coimmunoprecipitated with sgp130. The need of the site IIIc was unexpected based on the crystal structure of the vIL-6/gp130 complex or the IL-6/IL-6R/gp130 complex, since there is no direct contact of the amino acid residues constituting site IIIc and gp130 (8). In contrast, the newly identified site IIIc of vIL-6 alone was not sufficient for IL-6R-independent activation of gp130, since chimera IV5, which carries only the site IIIc of vIL-6 in the context of site IIIa and IIIb of IL-6 showed no activity in the absence of the IL-6R. Therefore, the transferred extended gp130-binding site III from vIL-6 to IL-6 appears to be the

minimal requirement for IL-6R-independent activation of gp130.

Although we have no experimental data, one might speculate that binding of hIL-6R to site I will induce a conformational change in site III and that this structural change may lead to a stable and active conformation only when the complete epitope is present, including site IIIc, which is not directly in contact with gp130. Such a "gp130-binding" conformation might be locked in the vIL-6 protein even without binding to the IL-6R. This situation is strikingly similar to chimeras between hIL-6 and CNTF, where the chimera IC5, which contained the CNTF residues corresponding to site IIIa and site IIIb (the top of helix A, the beginning of loop AB, together with the end of the CD loop and the top of helix D) was about 100 times less active than the chimera IC7, which in addition carried the sequences corresponding to the site IIIc of CNTF (19).

In summary, our results demonstrate that the functional/evolutional distance between hIL-6 and vIL-6 can be bridged by comparably small amino acid exchanges and that the extended binding site III but not binding site II is required for IL-6R-independent binding of vIL-6/IV9 to gp130. The successful constitution of an IL-6 chimera, which can bind to gp130 without the IL-6R, demonstrates that the recognition site of hematopoietic and neuropoietic cytokines can be regarded as discontinuous modules which could, in principle, be exchanged between different cytokines. The understanding of the α-receptor-independent interaction of vIL-6 is a starting point for the design of other cytokine variants that do not require α-receptors and might therefore exhibit a much broader spectrum of target cells than the parental cytokines. Such novel designer cytokines might be valuable for the stimulation of the immune system (33) and for ex vivo expansion of hematopoietic or other stem cells (1, 17, 40, 44).

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